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Combination of phage and Gram-positive bacterial display of human antibody repertoires enables isolation of functional high affinity binders

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Highlights

- First presented example of Gram-positive bacterial display of human scFv libraries
- Combination with phage display uses advantages of both platforms.
- Selected binders retained same affinity ranking in several formats.
- Suitable platform for affinity maturation

Abstract

Surface display couples genotype with a surface exposed phenotype and thereby allows screening of gene-encoded protein libraries for desired characteristics. Of the various display systems available, phage display is by far the most popular, mainly thanks to its ability to harbour large size libraries. Here, we describe the first use of a Gram-positive bacterial host for display of a library of human antibody genes which, when combined with phage display, provides ease of use for screening, sorting and ranking by flow cytometry. We demonstrate the utility of this method by identifying low nanomolar affinity scFv fragments towards human epidermal growth factor receptor 2 (HER2). The ranking and performance of the scFv isolated by flow sorting in surface-immobilised form was retained when expressed as soluble scFv and analysed by biolayer interferometry, as well as after expression as full-length antibodies in mammalian cells. We also demonstrate the possibility of using Gram-positive bacterial display to directly improve the affinity of the identified binders via an affinity maturation step using random mutagenesis and flow sorting. This combined approach has the potential for a more complete scan of the antibody repertoire and for affinity maturation of human antibody formats.

Keywords

S. carnosus, Flow cytometry, Antibody, HER2, phage display, cell-surface display, affinity maturation

Introduction

Antibodies have become indispensable tools as research reagents[1], in diagnostics[2] and as therapeutics[3–5]. To avoid immunisation of animals and to better tune the molecular characteristics of antibody reagents *in vitro*, several library-based methods mimicking natural evolution for screening and identification of antibody-antigen interactions have been developed[6]. Phage display[7,8] is currently the most widely used selection platform, its success mainly owing to its ability to conveniently harbour large libraries together with ease of handling. Other display formats include ribosome display[9,10] and cell based display platforms such as *E. coli*[11], yeast[12] and mammalian display[13]. The use of cell-based platforms offers several advantages compared to the capture and elution procedure of phage and ribosome display. Since cells, as opposed to phage, are large enough to be detected by light scatter in a flow cytometer, cells displaying a library of antibodies can be incubated with a fluorescently labeled antigen, allowing simultaneous real-time screening and selection. As the stringency of the sorting can easily be manipulated by changing the fluorescence gate, the discriminatory capacity and control of the system is superior[14]. In addition, the multivalent copies of affinity proteins displayed on the cells enable quantitative screening of relative affinities and selected clones can subsequently be characterised individually by flow cytometry without the need for sub-cloning.

Currently, several cell-based display systems are available. Yeast cells, being eukaryotic, possess glycosylation, disulphide-bridge forming and protein folding machineries similar to those of human cells. Differences, however, do exist between yeast and human processing[15] and glycosylation is typically not desirable during selection of scFv clones, as they will ultimately be reformatted and expressed as full-length antibodies in mammalian cell lines. Several display scaffolds derived from Gram-negative bacteria have also been employed in surface display[16,17], where *E. coli* is often the most commonly used host and its application in isolation of antibodies from libraries has been reported[18]. Recognising the capabilities of yeast and *E. coli* as display platforms, we believe it would also be interesting to utilise Gram-positive bacteria in the selection of antibody fragments from recombinant

libraries. In contrast with Gram-negatives, the outer layer of Gram-positive bacteria comprises only a single (plasma) membrane and one layer of peptidoglycan, making the translocation of anchored fusion proteins straightforward. The thicker peptidoglycan layer also acts as a physical barrier, protecting the cells from the harsh environment in the flow cytometer, leading to increased cell viability after sorting, a key requirement of cell surface display. Another key difference between Gram-negative and Gram-positive bacteria is the difficulty of transformation of the latter, which until lately[19] has made work in Gram-positive hosts difficult. Previous work has shown the potential of the Gram-positive *Staphylococcus carnosus* to be used in the selection of affinity proteins and enzymes of bacterial origin[20,21], cysteine-free camelid single domain antibodies[22], and for epitope mapping of linear[23] and structural epitopes[24]. *S. carnosus* surface display of a single murine scFv[25], as well as an attempt to express a library of several human multi-domain proteins and fragments[26], has further demonstrated the potential for this platform to display folded, functional, complex human proteins.

Building on previous experience of *S. carnosus* as a robust host for surface display and flow sorting, here we have further challenged it by using it as a platform for selection of human antibody fragments. The aim of this proof-of-principle study was to show the viability of this platform for selection and isolation of high affinity binders from antibody libraries. We demonstrate the first Gram-positive surface display of a human antibody library, by subcloning into *S. carnosus* a synthetic human scFv library pre-selected by phage display. We further show the combination of the two display methods by isolation of scFv with low nanomolar affinity against human epidermal growth factor receptor 2 (HER2) and demonstrate the usefulness of the Gram-positive platform as an efficient method for affinity maturation. We propose Gram-positive display as a convenient alternative for screening of moderately sized antibody libraries, such as those pre-enriched by phage display or affinity maturation libraries. We suggest that the combination of phage and Gram-positive bacterial display has the potential for a more complete scanning of the antibody repertoire than numerous individual ELISA screenings.

Materials and Methods

Phage display selections and characterisation of anti-HER2 scFv

Phage from HeL-11, a human synthetic scFv library[27], were cycled through rounds of selections with the extracellular domain of HER2 (Sino Biological, Beijing, PR China) as capture target. The antigen was covalently linked to magnetic beads with carboxylic acids using amine coupling according to the manufacturer's instructions (Dynabeads; Life Technologies, Carlsbad, CA, USA). Phage displaying nonspecific scFv were removed in a pre-selection step by incubating the library diluted in TPBSB (0.05% Tween 20 and 1% BSA in PBS) with the same type of bead but containing no antigen for 1h at room temperature (RT) and slow rotation. Subsequently the supernatant was transferred to the HER2-coated beads and similarly incubated for 1.5h. Following 4-7 rounds of washing in TPBS (0.05% Tween 20 in PBS) using a KingFisher Flex instrument (Thermo Scientific), the bound phages were eluted with trypsin, which cleaves at a trypsin-site located between the displayed scFv and protein III. The enzymatic activity was inhibited by the addition of aprotinin. Eluted phages were propagated by infection of *E. coli* XL1-Blue (Stratagene, La Jolla, CA, USA), infected with M13K07 (New England Biolabs, Beverly, MA, USA) for phage production and amplified in solution overnight at 30 °C. Harvested phage stocks were precipitated with polyethylene glycol (PEG), resuspended in PBS containing 0.1% BSA and used for subsequent rounds of selection. In total, three rounds of selections were carried out on gradually decreasing amounts of antigen and increasing number of washes.

Phagemid DNA from the third round of selection was isolated and recloned to allow production and analysis of soluble scFv. The DNA was digested with the restriction enzymes SfiI and AvrII (New England Biolabs, Ipswich, MA, USA) and ligated into the pHP2-15 vector[27] that provides the secreted scFv with three FLAG-epitopes and a hexahistidine tag at their C-termini. The constructs were transformed into Top10 *E. coli* (Thermo Fisher Scientific, Waltham, MA, USA). Single colonies were picked and soluble scFv produced in 96-well plates containing media supplemented with isopropyl thiogalactoside (IPTG) by incubation overnight at 37 °C and vigorous shaking (800 rpm). After sedimentation of the bacteria, the supernatants containing

the scFv were screened by enzyme linked immunosorbent assay (ELISA) for specific Her2 binding, using an HRP-labeled (horseradish peroxidase - labeled) anti-FLAG monoclonal antibody (Sigma Aldrich, St. Louis, MO, USA) as secondary conjugate. Clones that bound to HER2 but not BSA were subjected to DNA sequencing.

Cloning of human scFv and library into *S. carnosus*

All scFv constructs, i.e. individual clones as well as pre-enriched phage library pools, were sub-cloned into the Staphylococcal display vector pSCEM2 containing restriction sites NotI and AscI[26]. The scFv genes were amplified with PCR using primers containing NotI and AscI site handles and digested with respective restriction enzymes according to the manufacturer's instructions (Thermo Fisher Scientific). The genes were ligated into pSCEM2 and transformed into *E. coli* RR1ΔM15 cells. The single genes were prepared with QIAprep kit (Qiagen) and the gene library was prepared with a JETSTAR kit (Genomed, Bad Oeynhausen, Germany) according to the manufacturer's recommendations and transformed into electrocompetent *S. carnosus* TM300 using a previously described protocol[19]. The displayed scFv cassette on *S. carnosus* consisted of pro-peptide (PP) and signal peptide (S) at the N-terminal, followed by the scFv with an albumin binding protein (ABP) and a XM domain for cell wall anchoring at the C-terminal.

Error Prone PCR mutagenesis

Error prone mutagenesis of wild type clone SC1 was performed using GeneMorph II Random Mutagenesis Kit (Agilent Technologies, Santa Clara, CA, USA) in accordance with the manufacturer's instructions. In short, 1 ng plasmid DNA was used as the template and subjected to 25 cycles of PCR. The resulting library product was cloned into the vector pSCEM2 and transformed as described above.

Flow cytometry sorting of *S. carnosus* libraries (mock library, phage pre-enriched scFv library and error prone affinity maturation library).

An aliquot of 10 µL of the library corresponding to a number of cells ten times the size of the library was inoculated in TSB (tryptic soy broth) with 10 µg/ml chloramphenicol and cultivated at 37°C for 16 h at 150 <rpm. 10 µl of the overnight culture was washed with 500 µl PBS-P (PBS with 0.1% Pluronic acid (BASF)) and

pelleted by microcentrifugation (4000g, 4°C, 7 min) and resuspended in 200 µl PBS-P containing biotinylated antigen (200 nM and 100 nM in first and second rounds respectively) and incubated at room temperature under gentle mixing for 2 h. The cells were thereafter washed with 500 µl PBS-P and resuspended in 200 µl PBS-P containing streptavidin – R-phycoerythrin (rPE) (1 ng/ml; Life Technologies) and human serum albumin (HSA) – Alexa 647 (40 nM), followed by incubation on ice in the dark for 30 min. The cells were finally washed with 500 µl PBS-P and re-suspended in 500 µl PBS-P. The library was sorted using a MoFlo Astrios cell sorter (Beckman Coulter Inc., Brea, CA, USA). The gated cells were sorted into 200 µl TSB and incubated at 37°C for 2 h under gentle shaking and thereafter spread onto agar plates containing 10 µg/ml chloramphenicol. Colonies were screened using PCR and sequenced with BigDye thermocycle sequencing reactions and an ABI Prism 3700 instrument (Applied Biosystems, Foster City, CA, USA).

Flow cytometric analysis of single *S. carnosus* clones

S. carnosus cells expressing scFvs were cultivated in TSB medium with 10 µg/ml chloramphenicol for 16h at 37°C and 150 rpm. Cells were washed with 200 µl PBS-P (PBS with 0.1% Pluronic acid (BASF)) and pelleted by microcentrifugation (4000g, 4°C, 7 min) and re-suspended in 100 µl PBS-P containing 90 nM biotinylated antigen and incubated at RT under gentle mixing for 2 hours. After incubation, the cells were washed with 200 µl PBS-P and re-suspended in 100 µl PBS-P containing streptavidin – R-phycoerythrin (rPE) (1 ng/ml; Life Technologies, Carlsbad, CA, USA) and human serum albumin (HSA) – Alexa 647 (40 nM), followed by incubation on ice in the dark for 30 min. The cells were then washed with 200 µl PBS-P and re-suspended in 200 µl PBS-P, and analysed by flow cytometry using a Gallios Flow Cytometer (Beckman Coulter Inc., Brea, CA, USA).

Production of scFv binders as soluble molecules in *E. coli*

Chosen clones were amplified with PCR containing SfiI and AvrII restriction sites and digested with the corresponding enzymes, cloned into the pHP2-15 vector and transformed into TOP10 *E. coli* cells for expression of soluble molecules. Colonies were inoculated in Terrific Broth medium containing isopropyl thiogalactoside (IPTG) and soluble scFv produced overnight at 30°C. The proteins were purified from the periplasmic fraction making use of the hexahistidine tag and HisPur™ Ni-NTA

magnetic beads (Thermo Fisher) following the manufacturer's recommendation. Sample elution was performed in PBS containing 500 mM imidazole at pH8. The eluted scFvs were desalted using dialysis against PBS.

Analysis of scFv molecules on Octet RED96

To confirm successful selection and affinity maturation, the affinities of the two matured scFvs towards HER2 extracellular domain (ECD) in comparison with the non-matured scFvs were measured by biolayer interferometry (BLI) on an Octet RED96 system. Dip and Read™ Streptavidin (SA) Biosensors (ForteBio) were hydrated in assay buffer (0.1 mg/mL BSA, 0.002% Tween-20, PBS) according to the manufacturer's recommendations and conditioned by three alternating dips of 5 seconds each in regeneration buffer (10 mM glycine, pH 1) and assay buffer. Biotinylated HER2 ECD was immobilised on the hydrated biosensors at a concentration of 4 µg/mL. Baseline was established in assay buffer. Association of the scFvs was measured for 10 minutes in columns at five concentrations, from 400 to 25 nM for SC1 and SC1 error2, and from 200 to 12.5 nM for SC1 error4. Dissociation in assay buffer of the different scFvs was recorded for 10 min. An unrelated scFv binding to CTLA-4 was used as reference sensor. The biosensors were regenerated between the binding cycles as described above. K_D values were calculated based on both association and dissociation with a 1:1-binding model.

Reformatting scFvs as full length monoclonal antibodies

The V_H and V_L regions of each scFv and the two plasmids[28] used for mammalian production were amplified using 5' biotinylated primers (Integrated DNA Technologies, Coralville, IA, USA) designed with complementary regions of 30 bases for hybridisation. The PCR reactions for the plasmid and inserts were immobilised using 20 µg of Dynabeads® M-270 Streptavidin beads in 50 µl immobilisation buffer (2 M NaCl, 10% w/v PEG 6000, 10 mM Tris-HCl, 1mM EDTA, 0.1% Tween 20, pH 7.6). Plasmid and insert beads were resuspended in 20 µl 0.15 M NaOH for 10 min to allow for strand elution. Plasmid beads with ssDNA were captured and allowed to hybridise with eluted insert ssDNA in a mixture of 20 µl HCl (0.15 M), 10 µl Tris-HCl (1 M, pH 7.4), 10 µl PEG 6000 (50% w/v) and 20 µl water, preheated to 77 °C. The temperature was decreased by 1°C every 20 seconds until it reached 50°C,

followed by bead capture and strand extension. The extended constructs on the beads were released with FastDigest restriction enzyme and the plasmid containing solution was ligated using T4 ligase (ThermoFisher Scientific) according to the manufacturer's instructions. The ligation mix was finally used to transform *E. coli* for plasmid preparation.

Production of full length antibodies in HEK293T cells

Approximately 2×10^5 cells per transfection were seeded the day before in Dulbecco's Modified Eagle's Medium with 5% FBS (Sigma Aldrich, St. Louis, MO, USA). For each transfection, 2 μ g of heavy chain plasmid and 1 μ g of light chain plasmid were mixed with 6 μ g linear polyethylenimine (MW 25,000; Polysciences, PA, USA) in 200 μ l medium. The mixture was then added to the cells and the antibody containing supernatants were collected after 2 days.

Live cell binding analysis of reformatted full length antibodies

The relative antibody concentrations of the different sample supernatants were determined using quantitative ELISA Easy-Titer Human IgG (H+L) Assay Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. The different supernatants were then diluted to obtain the same relative antibody concentrations in all samples.

Human ovary adenocarcinoma (SKOV3) cells (LGC- lab standards AB, Borås, Sweden) with naturally over-expressed surface HER2, were cultivated in accordance with the manufacturer's recommendations, released from culture dishes with trypsin and washed once with PBS + 1% BSA (PBS:BSA). Approximately 2×10^5 cells were incubated with antibody containing supernatants with the following dilution factors: 1/1, 1/2, 1/4, 1/8, 1/16, 1/80, 1/400 and 1/2000 in 100 μ l reaction volumes for 2 hr at RT. The cells were washed with 200 μ l PBS:BSA followed by incubation with 1 ng/ml secondary antibody (goat-anti human H+L antibody – rPe conjugate; 1 ng/ml; Life Technologies, Carlsbad, CA, USA) in 70 μ l reaction volume with PBS:BSA for 30 minutes on ice. The cells were washed with 200 μ l PBS:BSA and re-suspended in 200 μ l in a sample tube. The binding abilities of the scFvs to SKOV3 cells were

evaluated with flow cytometry, using excitation at 560 nm and measuring fluorescence at FL2 after gating with respect to FSC and SSC.

Western blot analysis of reformatted full-length antibodies

For SDS-PAGE 1 mm precast NuPAGE Bis-Tris gels (Novex) were used in a 1x MES running buffer (50 mM MES, 50 mM Tris base, 3,5 mM SDS, 1 mM EDTA). The gel was loaded with 10 μ L sample and 6 μ L of SeeBlue Plus2 Protein Standard (ThermoFisher Scientific). Prior to loading, the protein sample was mixed 1:5 with 5x red loading buffer (5 mM EDTA, 433 mM SDS, 3,5 M β -mercaptoethanol, 100 mM Tris-HCl, bromophenol blue) and heated for five minutes at 95 °C. Electrophoresis was performed using a XCell SureLock Mini-Cell at a constant voltage of 190 V over 30 minutes. To transfer the proteins onto a membrane an XCell II™ blot module (ThermoFisher Scientific) was used. The PVDF membrane (ThermoFisher Scientific) was first activated in methanol and equilibrated in transfer buffer (25 mM Bicine, 25 mM BisTris, 0,8 mM EDTA, pH 7,2, 10% Ethanol). The transfer was performed at a constant voltage of 30 V for 90 min at 4 °C. The membrane was blocked with milk powder (5 % milk powder, 0,5 % Tween20 in 1x TBS-T) over night at 4 °C. The blot was incubated with goat anti-human horseradish peroxidase (HRP) conjugated antibody (ThermoFisher Scientific) diluted 1:5000 in blocking buffer for 1 hr at RT on a rotation mixer. After the first incubation step the blot was washed three times with TBS-T. To develop the blot the Immobilon Western Chemiluminescent horseradish peroxidase (HRP) Substrate (Merck Millipore, Billerica, MA, USA) was used according to the manufacturer's instructions. An image of the developed blot was captured using a BioRad CCD camera and software.

Results

As illustrated in **Figure 1**, we present a work scheme for display, selection and screening of human scFv libraries using *S. carnosus* display coupled to flow cytometric sorting, after initial enrichment by phage display. The scFv displayed on *S. carnosus* is preceded by a pro-peptide (PP) and signal peptide (S) at the N-terminal and has an albumin binding protein (ABP) and a XM domain for cell wall anchoring at the C-terminal (Figure 1A-B). During the selection process (Figure 1C), target-enriched phage-clones are transferred to *S. carnosus* and incubated with a biotinylated antigen. A second incubation with streptavidin-r-PE and labeled normalisation protein (HSA – 647) allows for two-colour fluorescent sorting with flow cytometry. The degree of binding strength to the antigen is determined by the magnitude of the signal from r-PE (Y-axis, signal FL2). The labeled normalisation protein binds to the ABP domain of the expression cassette and is used for normalisation and assessment of the expression level (X-axis, signal FL6). According to previous studies, the average expression level has been estimated to be about 10^4 to 10^5 recombinant proteins per cell[29]. Cells of interest, exhibiting high binding and surface expression (upper right corner) may be sorted by selecting a desired gate, amplified by overnight cultivation and either subjected to following rounds of sorting or isolation for characterisation as single clones.

Fluorescence detection and enrichment of cells expressing scFv on the surface of *S. carnosus* from a mock library.

Herceptin (Trastuzumab) in its full-length antibody form binds with high affinity to HER2. We cloned Herceptin as an scFv with a translocation signal for expression and anchoring to the surface of *S. carnosus*[30] under the control of a constitutive promoter (Figure 1). Transformed cells were detected in the flow cytometer by the size and granularity parameters, forward (FS) and side scatter (SS) (**Figure 2A**). HER2 binding was detected with biotinylated HER2 and streptavidin-rPE (FL2) whereas surface expression was detected with HSA – 647 (FL6). We observed a clear surface expression signal at 40 nM HSA – Alexa 647 for both Herceptin scFv (73% of cells within FL6 gate) and an anti-CTLA4 scFv (63% of cells within FL6 gate) (Figure 2B-C).

In the next step, we generated a mock library with Herceptin scFv clones spiked into a background of anti-CTLA4 scFv clones at a ratio of 1:10,000. The purpose of this experiment was to verify that the platform could be used to enrich positive binders against a target protein from negative binders in a library size similar to that typically obtained after initial pre-enrichment of a naïve library by phage. Flow cytometric cell sortings yielded enrichment after each round, and after two rounds of selection the ratio of Herceptin scFv to CTLA-4 scFv was 1:15 clones (Figure 2D-F). This shows the possibility of using FACS to enrich human scFv in Gram-positive display libraries.

Isolation of binders from pre-enriched antibody libraries using Gram-positive display

To further challenge the system, we used *S. carnosus* as a platform for library selection of novel antibody fragments specific for HER2. Prior to bacterial surface display, three rounds of phage selections on HER2 were performed using a human synthetic scFv library (Hell-11) of $>10^{10}$ members complexity[27]. The resulting library containing the scFvs from the third round of phage panning was cloned into the pSCEM2 vector and transformed into *S. carnosus* cells yielding a library of 10^4 transformants[23]. Screening and cytometric sorting of this library was first performed using 200 nM labeled HER2 and 40 nM HSA – 647 (**Figure 3A**). 3.8% of the population was gated and sorted as potential HER2-binding clones (Figure 3A, left). These sorted cells were expanded and subjected to an additional round of sorting under similar conditions (100 nM labeled HER2 and 40 nM HSA – 647) where 3.2% of the population was sorted (Figure 3A, middle). A clear enrichment could be seen following these two rounds of cytometric sorting (Figure 3A, right).

Characterisation of individual selected clones

We picked 96 positive single clones after two rounds of library sorting for on-cell flow cytometry analysis. The clones were ranked based on mean fluorescence intensity of antigen binding (FL2) while also taking surface expression (FL6) into consideration and 96 clones were sequenced of which 18 sequences were unique. The three best clones (SC1, SC39 and SC66) were chosen for a more detailed analysis. This involved flow cytometry analysis using scFv in surface displayed form (Figure 3B), as well as affinity determination by BLI using purified soluble scFv molecules

(Table 1) and live cell HER2 binding using scFv reformatted as full length monoclonal antibodies (Figure 3C).

Clonal on-cell flow cytometry analysis of the binders confirmed binding to native HER2 for all binders, with a slightly varying binding characteristics for each clone (Figure 3B). Cells were incubated with the same concentration of labeled HER2, followed by measurement of FL2 median antigen binding signal (Figure 3B right) in the gate containing surface expressed clones (Figure 3B left). The data shows that Herceptin in scFv-displayed form had the highest amount of bound HER2, as evidenced by the highest fluorescence signals. Taking only the antigen binding signal (FL2) into account, SC1 had the highest antigen binding of the three isolated clones followed by SC66 and SC39. This ranking remains when normalizing the antigen binding signal (FL2) to the surface expression signal (FL6) (Table 1, Supplementary Figure S1), but the stronger binding of SC66 compared to SC39 becomes more pronounced. As expected, the negative control CTLA-4 showed no binding.

Affinity measurements with scFvs produced as soluble proteins were performed using BLI (Table 1, Supplementary Figure S2). As a validation control, the affinity parameters of Herceptin scFv were determined ($K_D = 0.4$ nM; $k_{on} = 2.3E5$ M⁻¹s⁻¹; $k_{dis} = 9.8E-5$ s⁻¹), which were in agreement with other reported values[31–33]. The binder with the strongest relative target affinity when displayed on *S. carnosus* (SC1) also had the strongest affinity, with a K_D value of 12 nM. The measured K_D values of SC66 and SC39 were 14 nM and 300 nM respectively. The K_D values determined by BLI were consistent with the clonal ranking on staphylococcal cells (Figure 3B) and surface expression normalised antigen binding (Table 1, Supplementary Figure S1). Taken together these experiments suggest that Gram-positive display enables the isolation of high-affinity binders from a human antibody library.

Characterisation of selected clones reformatted as full length human monoclonal antibodies

Since functional full-length monoclonal antibodies are often desired for therapeutic purposes, the selected binders (SC1, SC39, SC66) were cloned as full length antibodies. Cloning of the scFvs into human constant region frameworks for both

heavy and light chains was performed using automated robot cloning[28]. The constructs were used to transfect HEK293T cells and a Western blot analysis was conducted to confirm that the monoclonal antibodies were produced in comparable amounts (Figure 3C, left). The antibody-containing medium supernatants were then used to assess binding on live SKOV3 cells using flow cytometry (Figure 3C, right). The antibody levels in each sample supernatant was first determined using ELISA and the different sample supernatants then adjusted for slight variations in antibody concentrations before incubation with SKOV3 cells and measurement of median fluorescence by flow cytometry. As expected, the full-length forms of Herceptin, SC1, SC66 and SC39 were all positive in SKOV3 binding with Herceptin having the highest signals. Furthermore, we observed the same relative ranking of binding strength between the samples when compared to the two previous assays (ranking on *S. carnosus* and using BLI). We could therefore conclude that the binding characteristics obtained from the clones in scFv format on *S. carnosus* are retained when reformatted into full-length antibodies.

Affinity maturation of wild type SC1 and isolation of mutants with enhanced affinities

One of the selected binders (SC1) was subjected to affinity maturation using error prone PCR. The resulting 10^4 library with an average of two mutated amino acids per gene, was sorted by flow-cytometry in a similar manner as the phage pre-enriched library (**Figure 4A**). Two isolated binders from the affinity maturation library (SC1M2, SC1M4) were again selected for detailed analysis. Clonal characterisation using both on cell flow cytometry analysis using scFv in surface anchored form (Figure 4B) as well as affinity characterisation using BLI of soluble scFv molecules (Table 1, Supplementary Fig. S2) showed improved binding for both affinity matured mutants, of which SC1M4 showed the largest improvement of affinity. The association and dissociation constants (Table 1) and the sensorgrams (Supplementary Figure S2) illustrate that this improvement was due to both a higher on- and a slower off-rate. Although SC1M2 appears very close to SC1 in the flow cytometry analysis (Figure 4B), when looking at the antigen binding signal (FL2) only, the improvement in affinity is clearer visualised when accounting for SC1M2's surface expression (Table 1, Supplementary Figure S3). This highlights the utility of the simultaneous monitoring of binding and surface expression for the ranking of clones. Both the

stronger binder SC1M4, with mutations in three positions, as well as SC1M2, with one mutation, had mutations located outside the CDRs (Supplementary Figure S4). Despite the modest improvement in affinity, we are able to demonstrate the possibility of using *S. carnosus* for affinity maturation of human scFv without the need for phage display.

Discussion

In this paper we present the first use of Gram-positive bacteria for display of human antibody libraries. We have shown that Gram-positive bacterial display can be used to isolate human scFvs with low nanomolar dissociation constants from a human antibody library pre-enriched by phage display. By combining bacterial display with phage display, we were able to use the advantages of both platforms. While phage display enables generation of larger libraries than bacterial display, the latter allows for thorough and fast screening and enrichment of binders using flow cytometry sorting, the equivalent of thousands of individual ELISA screening assays of *E. coli* expressed scFv proteins[34]. Furthermore, flow cytometric sorting enables the possibility of monitoring the binding process in real-time and gating a binding population for sorting of clones for subsequent rounds of selection and or downstream characterisation. A more stringent definition of the desired gate can be used to further minimise the sorting of non-binding clones. We have chosen to monitor fluorescent signal for both binding and surface display to allow sorting of clones based on their apparent affinity rather than their abundance on the cell surface. In the case of the three top binders identified by FACS here, none was present in an initial ELISA screening (47 clones) of the phage pre-selected library and only two (SC1 and SC66) were found after a more thorough ELISA screening (approx. 300 clones), whereas the third (SC39) could not be found. This was likely due to it being present in a much lower abundance and therefore requiring more time and effort to be successfully found by microtiter plate ELISA. Since the combination of phage display and deep sequencing allows for the time- and labour-efficient identification of rare binders[35,36], we believe it would also be interesting to combine phage display and deep sequencing with this screening platform to validate the functional binding of such a variety of clones.

By characterising the selected binders, we have also found that the relative binding ranking of the scFv displayed on *S. carnosus* is in line with the ranking of K_D values determined by BLI as well as ranking of binding to native HER2 present on SKOV3 cells using scFv reformatted as full length antibodies. Another potential benefit of selecting binders through a series of orthogonal selection systems could be the isolation of robust proteins with better general folding behaviour. This would be a desirable trait both as scFv or expressed as full-length proteins in a mammalian production host, as conversion of a phage display selected scFv into full length IgG may lead to a decrease in affinity[37]. In addition, switching expression system and affinity gating normalised on expression level could circumvent potential amplification or toxic biases that are sometimes observed in phage display systems[38].

As a final step, the staphylococcal display system was used successfully to affinity mature one of the earlier selected binders to show that this platform provides an efficient and streamlined approach to yield high-affinity binders. Although the mutations of both affinity matured clones were located outside the CDR-regions, we speculate that future usage of *S. carnosus* scFv libraries with diversity introduced in the CDRs using synthetic oligonucleotides is a viable alternative to error-prone PCR based libraries, with the potential to give an even better improvement in affinity.

In summary, we believe the technology we have developed here by combining phage display with staphylococcal display allows for a rapid and efficient generation of robust, high-affinity binders and aids the identification of low abundance library variants. Previous applications of this display platform together with observations noted in this study indicate that *S. carnosus* display can be utilised to complement existing technologies, particularly in combination with phage display. This is also the first time that human antibody fragments (scFv) have been subjected to library selection on Gram-positive bacteria. It would thus be interesting to perform selections against several other targets and compare the results with other platforms on a larger scale by utilising massively parallel sequencing methods[39–41].

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Author Contributions

F.J.H. and J.R. designed the experiments. F.J.H., A.L.V., H.P., A.S. and J.R. conducted the research. F.J.H., A.L.V., H.P. and J.R. wrote the manuscript. F.J.H., A.L.V., H.P., C.B., M.U. and J.R. reviewed the manuscript.

Additional Information

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Legends to figures

Figure 1.

A) Schematic representation of the staphylococcal cell surface. The fusion protein containing the pro-peptide (PP), the heavy (VH) and light chain domains (VL) of the antibody fused to an albumin binding protein (ABP) is anchored to the cell wall by the processed XM' domain. **B)** The pSCEM2 vector with the expression cassette used in this study. **C)** Selection procedure for fluorescent flow cytometric sorting of bacterial scFv libraries. (1) A human synthetic scFv library is pre-selected using phage display and cloned into *S. carnosus* cells. (2) Cells are incubated with labeled target and human serum albumin. (3) Clones are sorted using flow cytometry and the strongest binders (upper right corner, circled) are collected (4) for subsequent rounds (5) or characterisation.

Figure 2. FACS-based Enrichment of Herceptin scFv in a mock library of CTLA4 scFv clones.

A) Forward (FS) and side (SS) scatter dot plot showing detectability of *S. carnosus* displaying Herceptin scFv in a flow cytometer. Surface expression (FL6) monitoring of scFv construct was performed using HSA-Alexa 647 binding the ABP domain downstream of the scFv. Graphic profile of anti-CTLA4 **B)** and Herceptin **C)** scFv expression signal on the surface of *S. carnosus* as evidenced by FL6 signal. Detection of antigen binding (FL2) was performed using biotin labeled HER2 and streptavidin-rPE. A mock library with Herceptin scFv diluted in excess of an anti-CTLA4 scFv at 1:10,000 ratio was subjected to two rounds of sorting by gating for enhanced binding in dot plots **D-F)**. Enrichment of Herceptin scFv from the mock library can be clearly seen following two rounds of sorting **(F)** as evidenced by an increased population in the selected gate.

Figure 3. FACS sorting of anti-HER2 scFvs from pre-enriched library and characterisation of selected binders.

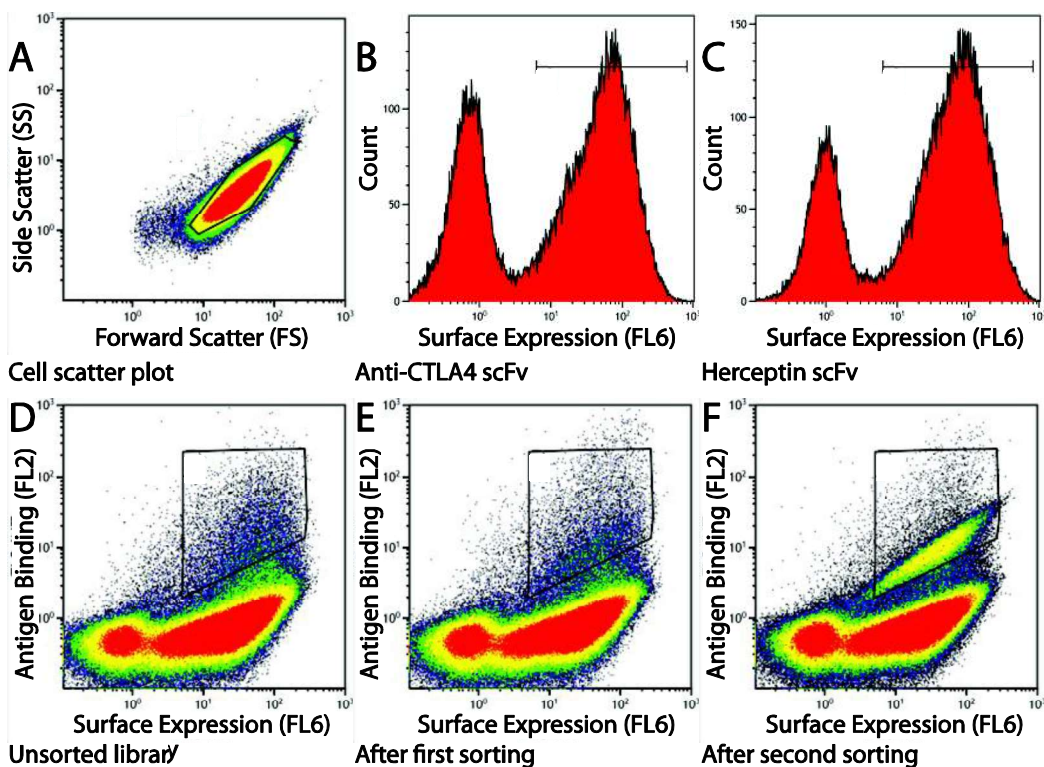
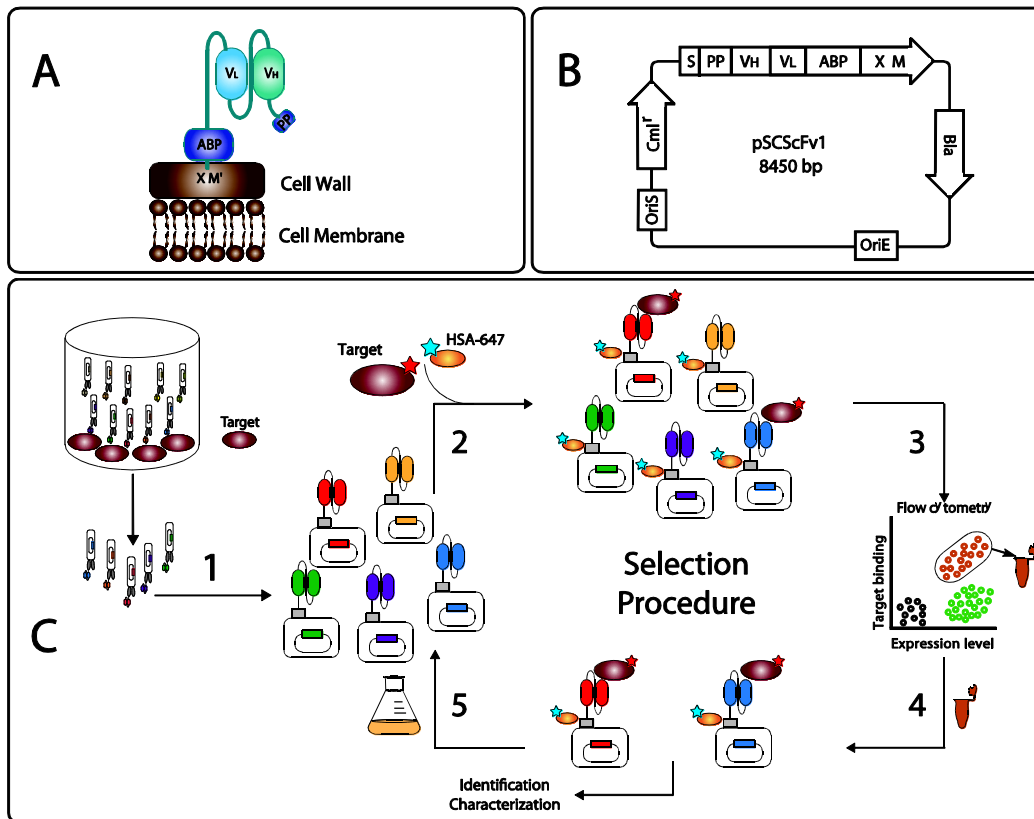
(A) Selection of scFv binders from a pre-enriched library against HER2 using FACS. A total of two rounds of sorting were performed whereby cells were sorted into the designated gate and subjected to another round of sorting (**A**). A clear enrichment was seen after two rounds of sorting (**A, rightmost panel**).

(B) Individual flow cytometry analysis of a selected scFv clone (SC1) displayed on *S. carnosus* (left panel). Flow cytometry overlay of scFv clones labeled with HER2 (right panel). An anti-CTLA4 and Herceptin in displayed form on *S. carnosus* were used as a negative and positive control respectively. ScFv clones selected by FACS sorting SC1, SC39 and SC66, exhibited different degrees of binding, as evidenced by the different MFI values.

(C) Analysis of scFvs reformatted to full-length antibodies was performed with Western blot (left) and flow cytometry binding assay to SKOV3 cells (right). Western blot analysis was performed on medium supernatants collected from transfected HEK293T cells (lanes 1-6: un-transfected cells; Herceptin; anti-CTLA4; SC1; SC39; SC66) to confirm the presence of the heavy and light chains of each antibody (C, left panel). HER2 expressing SKOV3 cells were incubated with antibodies listed. Anti-CTLA4 antibody (black) and Herceptin (red) were used as negative and positive controls respectively and all reformatted HER2-binders selected were able to bind SKOV3 to slightly varying degrees.

Figure 4. FACS sorting of error prone-PCR library allows isolation of affinity matured scFv

FACS sorting of affinity matured scFv binder after error prone PCR. The wild type SC1 was subjected to error prone PCR and flow cytometric sorting to find binders with enhanced affinity (**A**). The two unique binders that were selected (SC1 M2 and SC1 M4) were analysed using on-cell flow cytometry (**B**). The cells were incubated with labeled HER2, with the signal from HER2 binding shown on the X-axis (FL2) and frequency on the Y-axis. Both SC1 M2 and SC1 M4 have improved affinities against HER2.



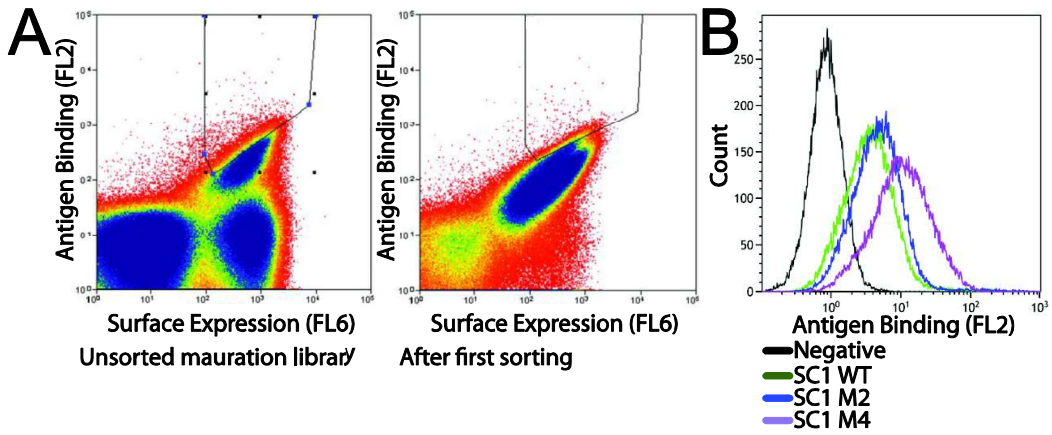
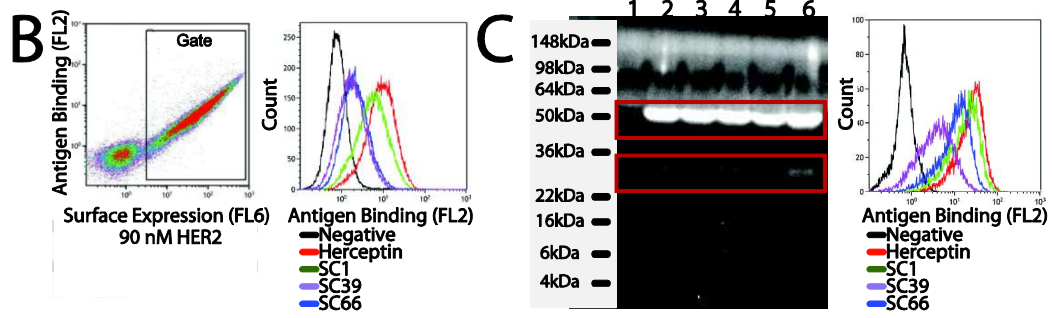
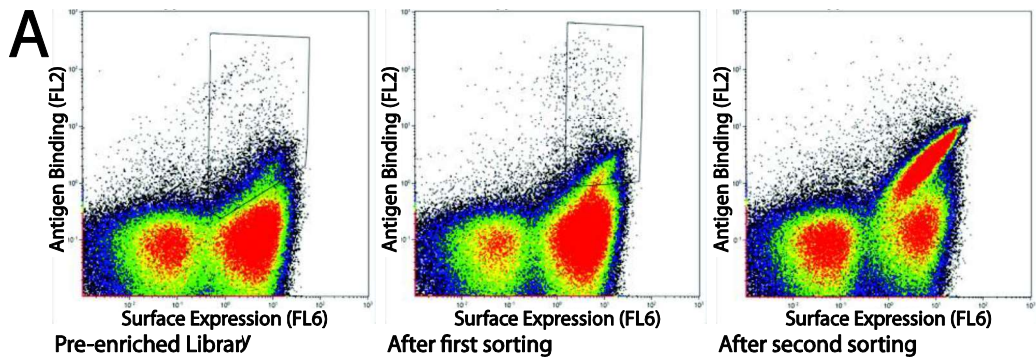


Table 1: Affinity Parameters

Name	K_D (M)	k_{on} (1/Ms)	k_{dis} (1/s)	Surface expression normalised antigen binding (FL2/FL6)
SC1	11.9 E-9 \pm 4.9 E-9	1.4E+05 \pm 5.6E+04	1.5E-03 \pm 7.1E-06	0.1
SC39	3.2 E-7 \pm 2.1 E-7	2.4E+04 \pm 2.4E+04	1.0E-02 \pm 1.0E-02	0.03
SC66	13.9 E-9 \pm 6.0 E-9	2.3E+05 \pm 1.1E+05	2.8E-03 \pm 1.4E-04	0.06
SC1M2	2.8 E-09 \pm 3.5 E-10	1.5E+05 \pm 7.8E+04	3.9E-04 \pm 1.7E-04	0.12
SC1M4	2.2 E-09 \pm 7.1 E-11	3.6E+05 \pm 1.3E+05	7.5E-04 \pm 2.5E-04	0.22